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Restriction Map of Corynebacteriophages β_c and β_{vir} and Physical Localization of the Diphtheria *tox* Operon

JOHN J. COSTA,† JAMES L. MICHEL, RINO RAPPUOLI,‡ AND JOHN R. MURPHY*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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The *Bam*HI, *Eco*RI, *Hin*dIII, and *Kpn*I restriction endonuclease maps of corynebacteriophage β_c and β_{vir} were constructed. β_{vir} appeared to be identical to β_c, except for an approximate 1-kilobase deletion that removed a *Bam*HI site, two *Kpn*I sites, and three *Eco*RI restriction sites. The diphtheria *tox* operon was located by hybridizing in vitro ^{32}P-labeled *tox* messenger ribonucleic acid to blots of endonuclease-digested β deoxyribonucleic acids. The messenger ribonucleic acid probe was found to hybridize to a 2.1-kilobase region of the β genome. Since approximately 1.9 kilobases is required to encode prodiphtheria toxin, the data presented strongly suggest that the *tox* operon of β is monocistronic.

Freeman (7) and Freeman and Morse (8) first described the lysogenic conversion of nontoxigenic *Corynebacterium diphtheriae* to toxigenicity after infection with the temperate corynephage β. Genetic evidence that a β-phage determinant was responsible for conversion to toxinogenesis was provided by Groman et al. (10-12). A partial genetic map of vegetative β phage has been described by Holmes and Barksdale (14), Matsuda et al. (17), and Singer (25). Although genetic analysis clearly demonstrated that β phage carried a *tox* determinant, it was not until Uchida et al. (28) isolated nontoxigenic mutants of β that *tox* was shown to be the structural gene for diphtheria toxin.

The prophage map of β is a circular permutation of the vegetative map (15). This observation suggested that linear β DNA has cohesive ends which allow the genome to circularize and to integrate into the corynebacterial chromosome according to the Campbell model (4). Additional support for this hypothesis came from electron microscopic examination of β DNA which revealed unit-length linear and circular molecules (3; Yin and Murphy, unpublished data).

Although the relative genetic map position of *tox* was known, its physical location on the β-phage genome was not. Physical localization of *tox* would allow for further study of interactions between postulated corynebacterial *tox* repressors and phage DNA (23). It would also facilitate the physical characterization of corynephage mutations, such as deletions or insertions, which may encompass both CRM$^+$ and CRM$^-$ phenotypes. Isolation of a DNA fragment containing the *tox* gene would make it possible to probe both toxigenic and nontoxigenic strains of *C. diphtheriae* for the presence of *tox* gene sequences. Finally, the determination of the physical location of *tox* is the first step toward the goal of elucidating its nucleotide sequence.

In this communication we demonstrate that β phage does contain cohesive ends. We present a restriction endonuclease map of corynephages β_c and β_{vir} and define the physical location of the diphtheria *tox* operon.

MATERIALS AND METHODS

Corynebacterial and corynephage strains. The clear plaque-forming mutant β_c and the virulent β_{vir} (24) were grown in the nonlysogenic, nontoxigenic *C. diphtheriae* $C7_s(-)^{tox^-}$ (1, 14, 19). mRNA was purified from the toxigenic PW8 strain as described previously (26).

Propagation and purification of β phage. Cultures of $C7_s(-)^{tox^-}$ were grown at 34°C in 10 liters of PT medium (20) in a Microferm fermentor (New Brunswick Scientific Co., Edison, N.J.) sparged with air at 0.5 liter/liter per min and agitated at 500 rpm. The pH was maintained at 7.4. Bacterial cultures were grown to an absorbance (at 590 nm) of 0.6 and then infected with β phage at a multiplicity of infection of 1 to 2. At the onset of mass cell lysis sodium citrate and $MgCl_2$ were added to 70 mM and 10 mM, respectively. Final β-phage titers ranged from 1×10^{10} to 7×10^{10} plaque-forming units per ml. Corynephage were harvested from crude cell lysates by the method of Yamamoto et al. (30).

DNA was released from purified phage by treatment with sodium dodecyl sulfate (21) and further

† Present address: Mount Sinai School of Medicine, New York, NY 10029.

‡ Present address: I.S.V.T., Sclavo, Research Center, 53100 Siena, Italy.

purified by phenol (redistilled under nitrogen) extraction. The yield of β DNA ranged between 1 to 5 mg/liter of culture. Purified DNA was exhaustively dialyzed against 10 mM Tris-acetate (pH 8.0) and stored over chloroform at 4°C until use.

Endonuclease restriction and gel electrophoresis. Purified β DNA was digested with restriction endonucleases *Ava*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Sal*I (New England Biolabs, Beverly, Mass.) according to the manufacturer's specifications. After digestion, samples were heated at 65°C for 5 to 30 min and transferred to an ice bath. DNA was electrophoresed in a vertical 1.3% agarose slab gel. Electrophoresis was performed in TEA buffer (50 mM Tris-hydrochloride [pH 8.0], 20 mM sodium acetate, 2 mM sodium EDTA, 18 mM NaCl) (13) for 4 to 6 h at a constant voltage of 70 to 80 V. Gels to be photographed were stained with ethidium bromide (2.5 μg/ml) for 30 min, illuminated with shortwave UV light, and photographed through a Hoya R(25A) filter on Polaroid type 47 film.

DNA ligation. *Escherichia coli* DNA ligase was purchased from New England Biolabs and used according to manufacturer's specifications.

Purification of DNA restriction fragments. Individual restriction fragments were purified by a modification of the method of Zain and Roberts (31). DNA, restricted with the same endonuclease, was electrophoresed in all wells of an agarose slab gel. After staining with methylene blue (0.05% wt/vol) for 1 to 2 h, bands were cut out of the gel. Gel slices containing identical fragments were placed in boiled 23-mm Spectraphor 1 (Spectrum Medical Industries Inc., Los Angeles, Calif.) dialysis tubing and covered with a minimal volume of TEA buffer. The dialysis bags were sealed and attached to a 60- by 0.8-cm glass rod which was placed into a vertical 70- by 2.5-cm cyclindrical electrophoresis chamber containing TEA buffer. The DNA was electroeluted from the agarose gel at 150 V for 5 h. Buffer containing the DNA was removed from the dialysis bag by using a silanized Pasteur pipette, and made 300 mM in sodium acetate, and the DNA was precipitated with absolute ethanol. After centrifugation (12,000 × *g* for 5 min), DNA pellets were dried under vacuum and suspended in DNA buffer (10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA) before use.

Partial purification of diphtheria *tox* mRNA from membrane-bound polysomes. The PW8 strain of *C. diphtheriae* was grown to an absorbance at 590 nm of 5 in C-Y medium, harvested at room temperature, and suspended in Chelex 100-treated C-Y medium to an absorbance at 590 nm of 8 to 10 (26). Samples (100 ml) were transferred to 2-liter Erlenmeyer flasks and incubated at 34°C with shaking at 240 rpm. Maximal toxin production began within 60 to 90 min and was monitored by rocket immunoelectrophoresis (20). At 120 min chloramphenicol was added to 200 μg/ml; the bacterial suspension was then poured over ice and harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. Pellets were frozen overnight at −70°C.

Bacterial pellets were thawed, and all additional work was performed at 4°C. Cells (5 g) were resuspended in 20 ml of 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM magnesium acetate containing 200 μg of chloramphenicol and 1 mg of heparin per ml. Bacteria were disrupted by passage through the French press at 20,000 lb/in^2, and electrophoretically pure DNase was then added to 5 μg/ml. Unbroken cells and debris were removed by centrifugation at 2,000 × *g* for 10 min. Membrane-bound polysomes were removed from soluble RNA by centrifugation (26). Pellets were resuspended in 1× SSC (15 mM sodium citrate, 150 mM NaCl, pH 7.0) containing 1% sodium dodecyl sulfate and warmed to room temperature.

RNA was extracted by the phenol-chloroform method of Palmiter (22). Before use all glassware was treated with Prosil-28 (PCR Research Chemicals Inc., Gainesville, Fla.) and baked (20). Endogenous RNases were removed from buffers by treatment with diethyl oxydiformate (DIP) (23); in some instances buffers were prepared with filtered, double-distilled water and autoclaved before use. Total RNA extracted from membrane-bound polysomes was precipitated with absolute ethanol, suspended in 300 mM sodium acetate, and stored at −70°C under ethanol until used.

Nucleic acid labeling and hybridization. DNA was transferred from agarose slab gels to nitrocellulose filter paper (Schleicher & Schuell Co., Keene, N.H.; type B85) by the method of Southern (27). β DNA was nick translated by using ^{32}P-labeled 5′-nucleotide triphosphates (>400 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (24). ^{32}P-labeled *tox* mRNA hybridization probes were prepared by dephosphorylating RNA isolated from membrane-bound polysomes with calf intestinal alkaline phophatase (Boehringer Mannheim) and then labeling the 5′ end with [^{32}P]ATP (1,000 to 3,000 Ci/mmol; New England Nuclear) and T4 polynucleotide kinase (Boehringer Mannheim) (6).

Hybridization of the [^{32}P]DNA probe to DNA on nitrocellulose filters was performed as described previously (9). The RNA probe was hybridized by the same method with the following modifications: 1× rather than 6× SSC and 5× rather than 1× Denhardt solution (5). *E. coli* tRNA was used as a carrier at 200 μg/ml.

[^{32}P]DNA (0.2 g, 5 × 10^6 cpm/μg) or RNA (0.2 g, 1 × 10^6 cpm/μg) was used for a nitrocellulose filter containing 2 to 10 digests. After extensive washings, the filters were autoradiographed on Kodak XR-1 film at −70°C for 3 to 96 h.

RESULTS

Localization of *cos*. The restriction endonuclease digestion patterns of β DNA were found to vary depending on how reaction mixtures were treated before electrophoresis. In the case of digestion with *Eco*RI or *Bam*HI, heating the samples to 65°C for 15 to 30 min before electrophoresis caused alterations in the relative intensities of ethidium bromide-stained bands as well as the appearance of new bands. Heating of *Eco*RI-digested β DNA reduced the staining intensity of band 3 (*Eco*RI-3), and gave rise to two additional bands, *Eco*RI-7 and *Eco*RI-8

(Fig. 1, lanes A and B). Analogous results were observed with *Bam*HI- (Fig. 1, lanes C and D) and *Sal*I- (data not shown) digested β_c DNA.

The simplest explanation for this observation is that the DNA fragments *Eco*RI-3 and *Bam*HI-2 contain the β-phage cohesive ends, *cos*, and that heating to 65°C causes a denaturation of this region which results in the appearance of the corresponding two smaller DNA fragments. The sums of the molecular weights of *Eco*RI-7 and *Eco*RI-8 and of *Bam*HI-3 and *Bam*HI-7 were found to be approximately equal to those of *Eco*RI-3 and *Bam*HI-2, respectively. To further demonstrate that *Eco*RI-3 and *Bam*HI-2 contained the *cos* region, β DNA was treated with DNA ligase before endonuclease digestion. As anticipated, after ligation the fragments *Eco*RI-3 and *Bam*HI-2 were resistant to heat denaturation (Fig. 1, lanes B and D). Analogous differences in the restriction patterns of ligated and unligated DNA were not observed when the endonucleases *Hin*dIII (Fig. 1, lanes E and F) and *Ava*I (data not shown) were used. These results suggest that *Hin*dIII and *Ava*I restriction sites are located close to, or within, the *cos* region.

Restriction endonuclease map of β_c and β_{vir}. The cohesive ends of β_c were used as a point of reference for physical mapping. The restriction map of β_c was determined by partial and limit digestion of purified DNA fragments with a second endonuclease. Figure 2 shows the restriction map of β_c with the *Eco*RI, *Bam*HI, *Hin*dIII, and *Kpn*I sites indicated. The molecular weights of each fragment and its digestion products were determined by using λ DNA digested with *Bam*HI and *Hin*dIII for molecular weight standards (Table 1). The initial partial restriction map was confirmed and completed by using purified ^{32}P-labeled β-DNA fragments obtained with each endonuclease to probe Southern blots of β_c and β_{vir} digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Kpn*I.

After completion of the restriction map of β_c, it was of interest to examine the restriction map of the closely related corynephage β_{vir}. Figure 3 shows a comparison of the *Eco*RI, *Bam*HI, and *Hin*dIII digestion patterns of these two corynephages. In the case of both *Eco*RI and *Bam*HI digestions, a novel large-molecular-weight DNA fragment was present and two smaller molecular weight fragments were missing in the ethidium bromide-stained digestion profile of β_{vir} as compared with that of β_c. For example, in the case of the *Eco*RI digest of β_{vir}, the DNA fragments corresponding to β_c *Eco*RI-5 and *Eco*RI-10 were missing, and a fragment larger than *Eco*RI-3 appeared. Digestion with *Hin*dIII, on the other hand, resulted in a slightly smaller β_{vir} *Hin*dIII-1 fragment (12.5 kilobases [kb]) as compared with the β_c *Hin*dIII-1 fragment (14.0 kb) (Table 1).

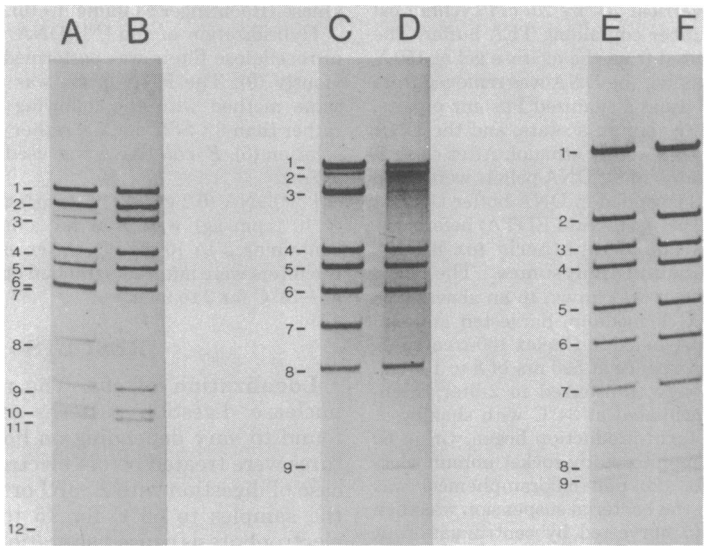

FIG. 1. *Restriction endonuclease digestion profiles of corynephage β_c DNA. The DNA in lanes B, D, and F was treated with DNA ligase before endonuclease digestion. After digestion all DNA samples were heated to 65°C and quenched on ice immediately before electrophoresis. Lanes: A and B, EcoRI; C and D, BamHI; E and F, HindIII. Ethidium bromide-stained DNA fragments are printed in the negative to intensify weakly fluorescent low-molecular-weight bands.*

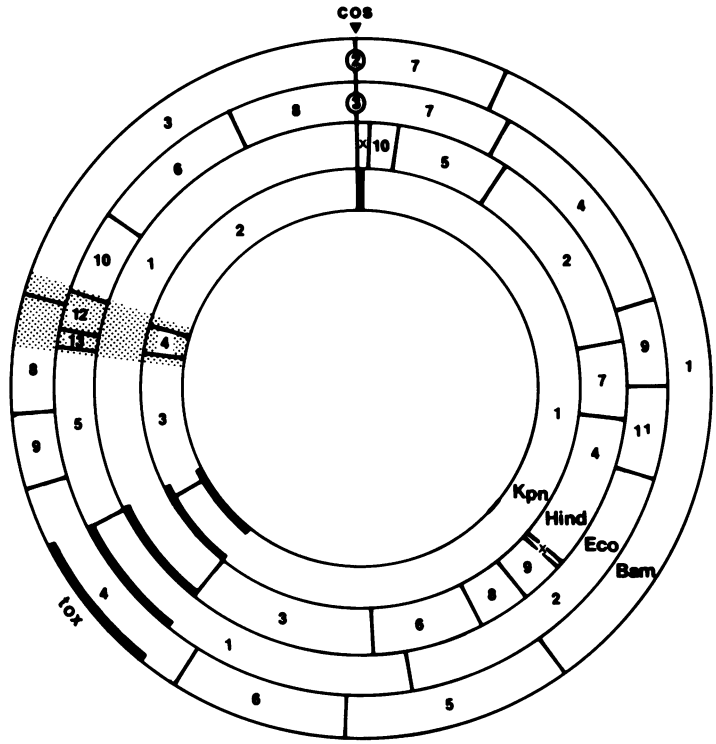

FIG. 2. *Restriction endonuclease digestion map of corynephage β_c. cos indicates cohesive ends, tox indicates diphtheria tox operon, and the stippled area indicates the deletion in corynephage β_{vir}.*

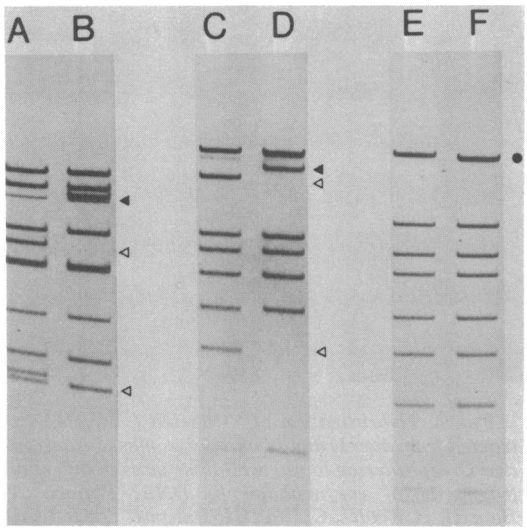

FIG. 3. *Comparison of the restriction endonuclease digestion profiles of corynephage β_c (lanes A, C, E) and β_{vir} (lanes B, D, F). The novel large-molecular-weight β_{vir} fragments (◀) and missing fragments (◁) in the EcoRI (lane B) and BamHI (lane D) digests are indicated. In the case of HindIII digestion of β_c DNA, the smaller HindIII-1 fragment is indicated (●).*

The simplest explanation for these results is that β_{vir} is a deletion mutant of corynephage β. Construction of the complete restriction map of β_{vir} shows that the *Eco*RI, *Bam*HI, and *Kpn*I restriction sites are missing (Fig. 2). The fusion of the adjacent DNA fragments would result in the appearance of the larger *Eco*RI and *Bam*HI bands (Fig. 3). Further support for this hypothesis comes from the comparison of the summation of β_c and β_{vir} restriction fragments. As can be seen in Table 1, the sum of the molecular weights of the *Eco*RI, *Bam*HI, and *Hin*dIII restriction fragments of β_{vir} is 600 to 1,500 base pairs smaller than that of β_c. The physical location of the deletion in β_{vir} is shown in Fig. 2.

Physical location of the diphtheria *tox* operon. Diphtheria toxin and its precursor have recently been shown to be the major polypeptides synthesized in vitro upon completion of nascent chains from membrane-bound polysomes of iron-limited *C. diphtheriae* PW8 (26). Therefore, membrane-bound polysomes were isolated from PW8 grown under conditions of either excess or limiting iron (20). RNA was purified by phenol-chloroform extraction and 5′ end labeled with [^{32}P]ATP as described above. The [^{32}P]RNA was then used to probe restriction digests of β DNA which had been trans-

TABLE 1. *Fragment sizes of β_c and β_{vir} DNA after endonuclease digestion*[a]

Band no.	*Bam*HI		*Eco*RI		*Hin*dIII		*Kpn*I	
	β_c	β_{ir}	β_c	β_{vir}	β_c	β_{vir}	β_c	β_{vir}
1	11.0	11.0	7.4	7.4	14.0	12.5	23.0	23.0
2	9.8[b]	11.0[b]	6.2	6.2	5.0	5.0	7.0[b, c]	10.5[b, c]
3	7.3[c]	8.5[c]	5.4[b]	5.8	3.8	3.8	3.5	<0.1[c]
4	3.9	3.9	3.9	5.4[b]	3.4	3.4	0.9	
5	3.5	3.5	3.5	3.9	2.6	2.6	<0.1[c]	
6	3.0	3.0	3.1	3.1	2.2	2.2		
7	2.5[c]	2.5[c]	3.0[c]	3.0[c]	1.7	1.7		
8	2.0	1.3	2.3[c]	2.3[c]	1.2	1.2		
9	1.3		1.8	1.8	1.1	1.1		
10			1.6	1.5	0.3	0.3		
11			1.5					
12			0.9					
13			0.2[d]					
X[e]					0.3	0.3		
Total	34.5	33.7	35.4	35.0	35.6	34.1	34.4	34.5

[a] Restriction endonuclease fragment sizes were determined from a plot of log electrophoretic mobility versus kilobase size by using *Bam*HI- and *Hin*dIII-digested coliphage λ DNA as a standard.

[b] DNA fragment containing an internal *cos* site.

[c] DNA fragment containing a terminal *cos* site.

[d] Detected only by Southern blot analysis of digested corynephage β_c DNA probed with intact ^{32}P-labeled β_c DNA or a ^{32}P-labeled restriction fragment.

[e] X refers to an undetermined number of DNA fragments of <0.1 kb.

ferred to nitrocellulose paper. As anticipated, only the RNA probe prepared from iron-limited cultures of PW8 was found to hybridize with restriction digests of β DNA (20, 26). As can be seen in Fig. 4, only one restriction fragment of DNA from each digest specifically hybridized with the RNA probe, except for the *Kpn*I digest. Analysis of the hybridization pattern revealed that these fragments included a common region of the β genome. The RNA probe hybridized to both the *Kpn*I-1 and *Kpn*I-3 fragments. Wallace et al. (29) have previously shown that small synthetic oligodeoxyribonucleotides (e.g., 17 base pairs) will specifically hybridize to the homologous region of the ϕ_X174 chromosome. Since only small regions of homology are required for detection by Southern blot analysis, we conclude that one end of the diphtheria *tox* transcript lies close to the restriction site between *Eco*RI-1 and *Eco*RI-5 (*Eco*RI-1–5). If the *tox* gene transcript extends into *Eco*RI-5, only a few base pairs must be present. The physical location of the *tox* operon on the β genome is shown in Fig. 2. Laird and Groman (16) and Holmes and Barksdale (14) have shown that the transcription of *tox* proceeds toward *att* in the prophage and vegetative phage genomes, respectively. Based upon the results of their genetic observations and our hybridization experiments, we would anticipate that the diphtheria *tox* promoter and regulatory loci are within the *Bam*HI-4 restriction fragment.

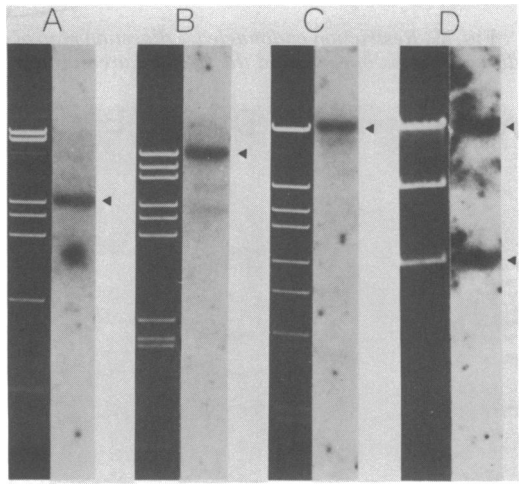

FIG. 4. *Hybridization of 5'-labeled [^{32}P]RNA extracted from membrane-bound polysomes of iron-limited C. diphtheriae to nitrocellulose blots of digestion fragments of corynephage β_c DNA. Panels: A, BamHI; B, EcoRI; C, HindIII; D, KpnI. The left side of each panel shows ethidium bromide-stained DNA fragments, and the right side shows the corresponding autoradiograph after hybridization.*

DISCUSSION

Using a series of corynebacteriophage β^{tox+}, γ^{tox-}, and L^{tox+} mutants, Holmes and Barksdale

(14) were able to define the relative genetic map order for the determinants of extended host range (*h* and *h'*), immunity (*imm*), and toxinogenicity (*tox*) on the vegetative genome. The genetic map of β was expanded by Matsuda et al. (17) and Singer (25) through the characterization of a series of temperature-sensitive (*ts*) mutations in cistrons coding for morphogenic determinants. On the vegetative map the *tox* marker is centrally located and is flanked on one side by cistrons involved in capsid and on the other by tail morphogenesis. Laird and Groman (15) have shown that the prophage map is a cyclic permutation of the vegetative map and that *tox* maps adjacent to *att*. This observation led to the suggestion that β integration into the bacterial chromosome proceeds according to the Campbell model for coliphage λ (4). The only direct evidence for cohesive ends on β DNA was electron microscopic observation of unit-length linear and circular molecules (3; Yin and Murphy, unpublished data).

The molecular weight of the β genome has been determined by electron micrograph contour length to be 22×10^6 (Wolfson and Dressler, quoted in reference 25) and 22.9×10^6 (3). In good agreement, we have found, by summation of molecular weights by restriction endonuclease fragments, the molecular weights of the β_c and β_{vir} genomes to be approximately 23.1×10^6 and 22.6×10^6, respectively.

In this paper we provide additional physical evidence for cohesive ends by the demonstration that ligation of β DNA before restriction endonuclease digestion stabilizes the *Eco*RI-3 and *Bam*HI-2 fragments. Without ligation these large bands are denatured at 65°C to their respective composite fragments. We observed that incubation at room temperature before electrophoretic separation of restriction fragments resulted in rapid reassociation of *cos*, suggesting a low activation energy for this region (Costa and Murphy, unpublished data). At present, the length of the β sticky ends and their nucleotide base sequence are not known. Nonetheless, the physical localization of *cos* provides a convenient orientation for the restriction endonuclease map.

The restriction endonuclease digestion pattern of β_{vir} is almost identical to that of β_c, with the exception of the *Eco*RI-5–13, *Eco*RI-13–12, and *Eco*RI-12–10 sites; the *Bam*HI-8–3 site; and the *Kpn*I3–4 and *Kpn*I-4–2 sites which are deleted in β_{vir}. The sum of the molecular sizes of the restriction fragments of β_c and β_{vir} and the differences in the molecular sizes of the corresponding fragments of β_c compared with the novel β_{vir} fragment obtained with *Eco*RI, *Bam*HI, and *Kpn*I digestion are consistent with an approximate 1,000-base-pair deletion in β_{vir}.

We have previously shown that only RNA extracted from the PW8 strain of *C. diphtheriae*, grown under conditions where *tox* was expressed, specifically hybridized to β DNA (20). Furthermore, we have shown that prodiphtheria toxin is synthesized in vitro upon completion of nascent polypeptide chains from membrane-bound polysomes (26) and that only RNA extracted from iron-limited cultures was active in synthesizing toxin-related polypeptides. In the present paper we used [^{32}P]ATP to in vitro end-label *tox* mRNA partially purified from membrane-bound polysomes of PW8 in vitro and used this preparation as a probe for the determination of the physical location of the diphtheria *tox* operon. As anticipated, only the RNA probe prepared from iron-limited cultures specifically hybridized to a common 2.1-kb region of β DNA which is contained within the 3.9-kb *Bam*HI-4 restriction fragment. The *tox* mRNA probe specifically hybridized to the 2.1-kb region between the *Eco*RI-5–1 site to the *Hin*dIII-1–3 site on the *Bam*HI-4 fragment (Fig. 2). The minimal size of the structural gene necessary for the expression of prodiphtheria toxin is approximately 1.9 kb (28). These two values are in good agreement and suggest that the *tox* promoter and regulatory loci may be carried on the *Bam*HI-4 fragment. Preliminary DNA binding experiments with *E. coli* RNA polymerase support the suggestion of a promoter on this DNA fragment (Michel and Murphy, unpublished data). The close agreement between the theoretical size of the *tox* structural gene and the size of the β DNA which specifically hybridized to the [^{32}P]RNA probe suggests that diphtheria *tox* *tox* operon codes only for the production of diphtheria toxin.

Although the genetic evidence strongly suggests that transcription of *tox* proceeds toward *att* (16, 18), the precise location of *att* on the vegetative map was not known. Laird and Groman (16) have presented two possible orientations of *imm* and *tox* on the prophage map which suggest that the vegetative gene order between *h* and *h'* may be either *h' imm tox att h* or *h' imm att tox h*. We have recently probed Southern blots of *Eco*RI-digested DNA extracted from lysogenic and nonlysogenic *C. diphtheriae* with ^{32}P-labeled β DNA and have positioned the *att* site in the approximate 1-kb region between the *Eco*RI-1–5 and *Bam*HI-4–9 restriction sites (Michel, Rappuoli, Murphy, and Pappenheimer, manuscript in preparation). These results indicate that the diphtheria *tox* promoter is close to the *Hin*dIII site on the *Bam*HI-4 fragment.

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ADDENDUM

While our work was in progress we became aware of the results of genetic studies in N. B. Groman's laboratory that were in agreement with the conclusion that the diphtheria *tox* operon is physically located on the *Bam*HI-4 fragment of corynephage β. An account of the results obtained by Buck and Groman is being published in this issue (2, 2a).

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